



fat where carotenoids (majorly lutein and/or  $\beta$ -carotene) are accumulated (Schweigert 1998). Pig, goat, sheep, and rodents belong to the first group, while cattle and horses are the main representatives of the second group, which also includes birds (Álvarez et al. 2015; Green and Fascetti 2016).

Scavenger species, especially vultures, have low concentrations of plasma carotenoids, which has been attributed to the scarce carotenoid content of rotten meat, together with other factors such as their relatively large size and scarce coloration (Tella et al. 2004). A previous study showed a nonlinear decrease in plasma carotenoid concentrations in relation to body size in obligate and facultative scavengers and predatory raptors, with the Egyptian vulture *Neophron percnopterus* and Andean condor *Vultur gryphus* deviating from the allometric scaling law (Blanco et al. 2014). These deviations were argued to be due to the exploitation of unusual food (herbivore excrements and vegetal matter), as an adaptation compensating for low carotenoids content in the decomposing carcasses of the vertebrates that primarily constitute their diets, despite a comparatively high demand for these micronutrients and pigments for coloration (Negro et al. 2002; Blanco et al. 2013; Plaza et al. 2020a). The consumption of unusual foods can be self-regulated by adult individuals searching for and ingesting this matter depending on their requirements for health, reproduction, and coloration, while nestlings must rely on parental decisions regarding provisioning. The study of specific genes and mutations relevant to explaining broad patterns of metabolism and coloration of carotenoids in animals has advanced enormously in the last decades (Lopes et al. 2016; Toews et al. 2017). These studies require prior baseline information on the ecology and genomics of the species under study, which in the case of vultures is very limited or nonexistent.

Several studies have highlighted the importance of environmental factors to explain carotenoid profiles and their influence on nestling coloration, particularly the availability and accessibility of carotenoids in food (Negro et al. 2000; Isaksson and Andersson 2007; Ewen et al., 2008). Traits of individual nestlings, such as sex, age, position in competitive brood hierarchies, and health can also contribute to their physiological and competitive abilities to uptake carotenoids from their usual diet (Bortolotti et al. 2000; Sternalski et al. 2010). Nestlings of many raptor species show a less colorful appearance than adults, and this difference may last several years during the prebreeding stage of young individuals. This delayed coloration often includes carotenoid-dependent expression in the integumentary system, especially in the face, cerea, and legs (hereafter integument), and increases in brightness and hue with age and health, reaching its maximum expression when reaching breeding age (Negro et al. 1998; McDonald 2003; Blas et al. 2013). Whether these differences depend on contrasting diets between nestlings and adults, or due to the development of physiological processes for the intake and assimilation of carotenoids for deposition in the integument, or other functions, remain unclear. In addition, no information is available on the parental provisioning of unusual food to offspring, including fresh plants, feces, and other matter, that could explain potential differences in the carotenoid content in nestlings of different species. Therefore, nestlings can be good study models to evaluate the factors behind carotenoid intake and use between related species, particularly to examine differences in their diet and coloration, as well as for intraspecific studies comparing diets between areas and between nestlings and full-grown individuals.

In this study, we determined the occurrence of carotenoids and their concentrations in the plasma of the nestlings of cinereous vultures *Aegypius monachus*, griffon vultures *Gyps fulvus*, and Egyptian vultures living in sympatry. We evaluated nestling diet and parental provisioning of unusual sources of carotenoids (fresh vegetal matter and vertebrate feces), and reviewed the information on carotenoid content in different tissues of livestock species that constitute the main diet of vultures and other scavenger species. We aimed to explore two not-mutually exclusive hypotheses on carotenoids availability, uptake, and expression in nestling integument coloration. The “pigment availability” hypothesis predicts that the profile of plasma carotenoids should include a higher variety of compounds at higher concentrations in species with a more varied diet that includes food with a higher carotenoid content (Tella et al. 2004; McGraw 2005; Olson 2006), or by ingesting unusual matter rich in carotenoids (Blanco et al. 2013). Because carotenoids intake can vary in relation to the need for different functions (Britton et al. 2008; Bohn et al., 2017; Widjaja-Adhi and Golczak 2020), this hypothesis has no clear specific predictions connecting carotenoid availability with use for different functions. In fact, carotenoid assimilation for interrelated coloration expression and health may ultimately depend on the performance of the physiological machinery to uptake, transport, metabolize, store, and deposit these compounds (Yang et al. 2020), and be modulated by breeding status, sex, size, parasites, pollutants, activity, and other factors (Bortolotti et al. 2000; Blas et al. 2006, 2013; Leclaire et al. 2019; Alonso-Alvarez et al. 2022). While these factors have been evaluated intraspecifically, a similar reasoning can apply to comparisons between related species with a relatively similar diet, like vultures, where differences in carotenoids metabolism are expected to depend on species-specific traits such as selective absorption processes in the small intestine, the occurrence and action of transport proteins, and other factors (Furr and Clark 1997; Tella et al. 2004; Alonso-Alvarez et al. 2022). The “pigment assimilation-signaling” hypothesis posits specific physiological mechanisms to efficiently use these micronutrients for color signaling rather than depending only on carotenoid availability and uptake (Tella et al. 2004; Fernández-García et al. 2012; Widjaja-Adhi and Golczak 2020). This hypothesis predicts a higher variety of pigments assimilated at higher concentrations from a similar diet, including from unusual sources of carotenoids, in species with carotenoid-dependent coloration, even when these pigments are not deposited in the integumentary system of nestlings.

## Material and Methods

### Study area and species

The study was conducted in the Central Range Mountains, which includes habitats such as montane pinewoods and canyons in the Castilian Highlands, in Segovia, Ávila, and Madrid provinces, central Spain. The cinereous vulture population consists of about 200 pairs with a relatively stable population trend in recent years, following two decades of population recovery in the study area (Donázar et al. 2002, own data). In this area, cinereous vultures nest in montane pinewoods (Fargallo et al. 1998; Chakarov and Blanco, 2021), and forage on carcasses of wild rabbits *Oryctolagus cuniculus*, wild ungulates, and livestock (Blanco et al. 2017, 2019). The griffon vulture population remains relatively stable at about 2,500 breeding pairs after a long period of

population increase that was interrupted by the mad-cow crisis between 2002 and 2011 (Almaraz et al. 2022). Most pairs nest on rocky outcrops and rarely on pines (Chakarov and Blanco 2021), and forage mainly on carcasses of stabled livestock, mostly swine and poultry, found at carcass dumps (Blanco 2014, 2018). The Egyptian vulture in the study area is limited to the breeding population in the Segovia province. In this area, a small and declining population of about 30 pairs nests mostly in large gorges located in two protected areas (Natural Parks of Hoces del Río Duratón and Hoces del Río Riaza), with isolated pairs nesting in the surrounding areas (Blanco and Morinha 2021; Serrano et al. 2021). The diet of Egyptian vultures is comparatively more diverse than those of the other 2 species, and includes a variety of wild vertebrates and livestock carcasses (Blanco et al. 2017, 2019).

Nestlings and adults are sexually monochromatic in the three vulture species. Cinereous and griffon vultures have black and brown plumage, respectively, with differences in hue between individual griffon nestlings (Fargallo et al. 2018) and more so in adults due to plumage clearing with age (Elósegui 1989, Mundy et al. 1992, Figure 1A–C). Exposed skin on the head, ceres, and legs varies from pinkish white to gray, with slight differences between adults and nestlings (Figure 1A–C), while the iris color varies from dark brown to black and clears with age to light brown and gray in the cinereous and griffon vulture, respectively. Adult cinereous vultures have a slight blue hue in the ceres (Figure 1A). The dark plumage of Egyptian vulture nestlings is progressively replaced by a white plumage that is definitive from the fourth year of life (Donazar 1993, Figure 1d). Nestling Egyptian vultures have variations in individual plumage coloration, varying from pale brown to black (Figure 1E). There is no evidence of carotenoid expression in the coloring of the integument of nestlings in any of the three species, nor in adult cinereous and griffon vultures. On this basis, we can reasonably assume

that both adult and nestling cinereous and griffon vultures do not trade off allocation of carotenoids between body maintenance and health signaling or ornamentation. Only adult Egyptian vultures show carotenoid-dependent coloration, expressed in their characteristic orange–yellow face (Negro et al. 2002, Figure 1D) which develops simultaneously with the development of definitive white plumage (Donazar 1993).

### Nestling sampling and diet

Nests were regularly monitored by telescope to determine breeding success and estimate the adequate sampling date of nestlings, following standard methods (Martinez et al. 1997; Donazar et al. 2002; Sanz-Aguilar et al. 2017). When nestlings were feathered but prior to the time when they are ready to fly (about 40 days old in the Egyptian vulture, and about 70 days old in cinereous and griffon vultures), their nests were accessed by climbing to band, measure wing length (to the nearest mm) as a surrogate of age, and to take blood samples (3–5 mL) from the brachial vein (details in López-Rull et al. 2015; Chakarov and Blanco 2021). Blood was conserved with dry heparin and transported in a cooler, and centrifuged at 13,000 g for 10 min in the laboratory to obtain plasma, which was frozen at  $-20^{\circ}\text{C}$  until analysis. A small quantity of blood was used for sexing individuals through molecular procedures after DNA extraction (Gómez-López et al. 2022). The nestlings analyzed for carotenoid levels were sampled in 2013 (griffon vulture,  $n = 35$ , cinereous vulture,  $n = 5$ , Egyptian vulture,  $n = 11$ ) and 2004 (cinereous vulture,  $n = 5$ ). Since griffon vultures and cinereous vultures only raise one nestling per year, the sample size of nestlings corresponds to the number of sampled nests. In the case of the Egyptian vulture, which can raise two sibling nestlings each year, the number of nestlings corresponds to 8 nests (5 of one nestling, and 3 of two nestlings).

We compiled published information on nestling diet from 2003 to 2017 (Blanco et al. 2017, 2019), and complemented



**Figure 1** Photographs showing the physical characteristics of the study species. (A) Adult cinereous (left) and griffon (right) vultures (credit: Jorge de la Cruz); (B) nestling cinereous vulture (credit: Armando González); (C) nestling griffon vulture (credit: Armando González); (D) adult Egyptian vulture (credit: Jorge de la Cruz); (E) nestling Egyptian vultures (credit: Jonathan García).

it with additional data from the three vulture species in the study area collected afterward until the breeding season of 2022. Remains from animal food carcasses and pellets found in and below the nests were collected at the time of nestling banding. The method of diet quantification was the same as in the previous studies cited above. Briefly, we considered all types of remains found in the nests, which differed in frequency among the species studied (Donázar 1993), to take advantage of the information provided by each type of rest, so that the quantification of the diet was as general as possible by using complementary study methods, which aimed to reduce the biases inherent to each of them (Donázar et al. 2010). Carcass remains were identified to species level and quantified assuming the smallest possible number of individuals according to their size, age, and anatomical position. These remains are particularly numerous in the case of Egyptian vultures, which accumulate bones, feathers, and other remains of wild and domestic animals transported to the nest individually in their beaks (Donázar 1993). In the case of cinereous vultures, the pellets found in the nests were also taken into account, as food remains are often scarce or lacking. Bones, hair, and feathers were identified at species level using reference collections. The presence of each animal species was recorded qualitatively in each pellet given the difficulty of quantifying the corresponding number of carcasses (Donázar et al. 2010). Consequently, to avoid pseudoreplication, the presence of identifiable remains of each species found in the pellets was considered only when no food remains (bones, skins) of the same food species were found in the same nests during our snapshot sampling. In the case of griffon vultures, the remains vomited by the nestlings during handling were identified by looking specifically for the presence of identifiable bones, hair, and feathers. The pellets found in the nests of griffon vultures were also analyzed following the methodology used for the cinereous vulture, as they correspond to a time period prior to that of the vomiting. Animal food species were identified, quantified, and categorized as wild reptiles and amphibians, lagomorphs, other mammals, birds and fishes, or domestic animals, categorized as sheep and goats, cattle and horses, pigs, domestic rabbits, poultry, and other food including remains of domestic refuse, following Blanco et al. (2019). Since vultures generally divide carcasses among several individuals, either intra- or interspecifically, and because they chop up carcasses in order to transport some remains to the nest, it is not feasible to calculate the biomass contributed by each type of food to estimate the diet of the nestlings from the remains found in the nests (Hiraldo 1977; Donázar 1993). The analysis of pellets neither allows the estimation of the biomass corresponding to each food species because (1) the different vulture species feed on different parts of the same carcass, for example, griffon vultures on entire carcasses including viscera, cinereous mostly on skin, cartilage, and tendons, and Egyptian vultures mostly on flesh (Donázar 1993). These differences are reflected in the indigestible remains in the pellets in a way that may be more evident in the cinereous vulture than in the other two species; (2) pellet production does not appear to show a regular pattern due to the intermittent feeding of the nestlings, and because pellet production is affected by the type and amount of indigestible material, especially hair, feathers, plant material, and, to a lesser extent, bones; (3) the amount of food provided to the nestlings may depend on multiple factors that cannot be controlled for in order to estimate the biomass provided from each food species. For example, parents may

feed their nestlings with the remains of several animal species mixed in the crop (e.g. pig and poultry carcasses exploited in the same carcass dump), but it is challenging to know their proportion, or the contribution of each feed to the production of pellets. Sometimes, the remains of some livestock species correspond to viscera that do not allow a correct identification in vomits and become lacking in pellets, while in other cases, they correspond to whole carcasses that leave visible remains (e.g. poultry feathers) in the vomits, as well as indigestible remains in pellets; (4) vultures often use carcasses that have been previously fragmented (e.g. from roadkill or slaughterhouse), semi-consumed by other scavengers, or in varying degrees of decomposition, which prevents a proper estimation of the biomass attributed to the remains found in the nests.

### Vegetal matter in nests and pellets

The presence of fresh vegetal matter in the nests was recorded during nestling banding from 2003 to 2022. During the breeding season of 2017–2022, a more detailed evaluation of the occurrence of vegetal matter in the nests was conducted. We specifically recorded whether fresh vegetal matter recently provisioned corresponded to the plant typology and species used to build the main supporting nest structure or the inner lining (Fernández and Fernández 1974; Xirouchakis and Mylonas 2007), and whether it could be provided for the nestling consumption as an unusual source of carotenoids. To try to distinguish between these functions, we recorded if the provisioned vegetal matter was part of the lining of the nest that the parent vultures could arrange as a way to sanitize the nest, for example, as drying material or to try to avoid the accumulation of dirt or the presence of parasites (Xirouchakis and Mylonas 2007; Kushwaha 2015, 2016). This material is assumed to fulfill this function when it corresponds to woody material of the same structure as that which forms the nest or its inner lining (Fernández and Fernández 1974; Xirouchakis and Mylonas 2007). We considered that the green material could be brought into the nest by the parents for potential consumption by the nestling (authors' pers. obs.) when it refers to vegetal matter of nonwoody structure that could apparently be ingested without difficulty, as evidenced by green material present in the vomit regurgitated by the nestlings during handling. This differentiation refers specifically to the griffon vulture, as cinereous vulture and Egyptian vulture nestlings generally do not vomit during handling.

In addition, we determined the presence of vegetal matter in the pellets of the three vulture species found in each nest during the sampling period. We searched for the presence of vegetal matter, and recorded when it was distributed throughout the pellet, shaping its structure, which suggests active ingestion of plant matter or its passive or active ingestion from the digestive tract of carcasses, but not when it appeared in residual form as a few small fragments hardly visible inside the pellet.

### Livestock feces as a source of carotenoids

The presence of feces from livestock and other animals, including domestic and wild species, was recorded in each nest. The objective of this sampling was to evaluate if parental vultures could be supplying their nestlings with this matter as a food source of carotenoids.

To evaluate the presence and concentration of carotenoid pigments in feces from livestock, we collected fresh feces of cattle ( $n = 10$ ) and sheep ( $n = 10$ ) in the study area. The feces of these species were selected because they are the most

available and abundant in the foraging areas of the vulture species, and because the carcasses of these free-grazing livestock are exploited by the three vulture species in the study area. The feces of indoor-farmed pigs and poultry were not considered, as these species are fed with industrial feed rather than with plants, and because vultures do not have access to recently excreted feces at the intensive farms where they are raised. Therefore, it is not to be expected that vultures can provide excrements of these species obtained after excretion by the animals in the field, as an active form of supplying the nestlings with potentially carotenoid-rich material.

### Extraction, identification, and quantitative analysis of carotenoids

Carotenoids were extracted from plasma samples as described previously (Blanco et al. 2005). In brief, a plasma aliquot (100  $\mu$ L) was lyophilized and the carotenoid pigments were extracted from the dry residue with 200  $\mu$ L of N,N-dimethylformamide (NNDMF) after a 60-min period that included sonication for 5 min every 30 min. The resulting extract was analyzed by high-performance liquid chromatography (HPLC) in accordance with the procedure of Mínguez-Mosquera and Hornero-Méndez (1993) with some modifications (Blanco et al. 2013). For measurement of carotenoids in feces, 5 g of feces were extracted with 5 mL of NNDMF with sonication for 10 min and subsequently with continuous shaking for 120 min. The resulting extract was centrifuged at 12,000  $\times$  g for 5 min and the upper layer was stored at  $-30$  °C until analyzed by HPLC. The chromatographic analysis was carried out on the same day as the preparation of the extracts. Analyses were carried out in duplicate. All operations were carried out under dimmed light to prevent isomerization and photodegradation of carotenoids.

The identification of the carotenoid pigments present in plasma and feces was carried out following standard procedures (Schiedt and Liaaen-Jensen 1995), including co-chromatography with standards, acquisition of UV-visible spectra, as well as chemical derivatization microscale tests for the examination of functional groups (5,6-epoxide, hydroxyl, and carbonyl) (Eugster 1995). The chromatographic, spectroscopic, and chemical properties of the pigments were compared with authentic carotenoid samples and with the data available in the literature (Britton 1991, 1995; Britton et al. 2004). Authentic pigment samples of carotenoids were isolated from natural sources:  $\beta$ -carotene ( $\beta,\beta$ -carotene),  $\beta$ -cryptoxanthin ( $\beta,\beta$ -caroten-3-ol), zeaxanthin ( $\beta,\beta$ -carotene-3,3'-diol) were obtained from red peppers (*Capsicum annuum* L.), lutein ( $\beta,\epsilon$ -carotene-3,3'-diol) from mint leaves (*Mentha arvensis* L.), lycopene ( $\psi,\psi$ -carotene) from tomatoes (*Solanum lycopersicum* L.) and zeinoxanthin ( $\beta,\epsilon$ -caroten-3-ol) from orange juice. Canthaxanthin ( $\beta,\beta$ -carotene-4,4'-dione) was purchased from Sigma-Aldrich, and the echinenone standard was a generous gift from Dr. George Britton (School of Biological Sciences, University of Liverpool, UK). Reference material was not available for  $\gamma$ -carotene, and therefore, this pigment was tentatively identified and marked as “ $\gamma$ -carotene-like.” The identification of *cis* isomers of lutein and zeaxanthin was based on the presence and relative intensity ( $\%A_B/A_{II}$ ) of the *cis* peak at about 330–340 nm in the UV-visible spectrum, a reduction in the fine structure and a small hypsochromic shift in  $\lambda_{max}$  with respect to the all-*trans*-lutein, and the chromatographic behavior in the C18 reversed-phase HPLC column (the *cis* isomers are slightly more retained than the all-*trans*

isomer) (Britton 1995). For the sake of simplicity, geometrical isomerism (*cis/trans*) was only specified for the *cis* isomers, the rest being considered all-*trans*.

Quantitative analysis of carotenoids was carried out by HPLC according to the method of Mínguez-Mosquera and Hornero-Méndez (1993) with some modifications. The HPLC system consisted of a Waters 2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector, and controlled with Empower2 software (Waters Cromatografía, S.A., Barcelona, Spain). A C18 reversed-phase analytical column (Mediterranea SEA18, 200 mm  $\times$  4.6 mm i.d., 3  $\mu$ m; Teknokroma, Barcelona, Spain) was used. Separation was achieved by a binary-gradient elution using an initial composition of 75% acetone and 25% deionized water, which was increased linearly to 95% acetone in 10 min, then raised to 100% in 2 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. An injection volume of 20  $\mu$ L and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra were acquired in the 350–700 nm wavelength range. Quantification was carried out using external standard calibration curves prepared with zeaxanthin, lutein, canthaxanthin, echinenone,  $\beta$ -cryptoxanthin,  $\beta$ -carotene, and lycopene standards. Calibration curves were prepared in the range of 0.5–50.0  $\mu$ g/mL, and constructed by plotting the peak area at 450 nm versus the pigment concentration. The calibration curves of all-*trans*- isomers were also used to determine the concentration of the *cis* isomers.

### Statistical analyses

Between-species differences in the concentration of each plasma carotenoid were evaluated with one-way ANOVAs and post hoc Tukey tests. Data were  $\log_{10}$  transformed to attain normality. The Kruskal–Wallis and Mann–Whitney tests were used for non-normal data. Pair-wise relationships between the concentrations of each type of plasma carotenoid found in each individual were evaluated using Spearman correlations.

The variation in total plasma concentration (mg/mL) of xanthophylls, carotenes, and total carotenoids according to age and sex were evaluated for each vulture species by using generalized linear models (GLMs) with Gaussian error distribution and identity link function. Wing length was used as a surrogate of age in the growing nestlings (Elósegui 1989; Gómez-López et al. 2022).

Statistical analyses and model validation were performed using SPSS software v. 28 (IBM Corp., Armonk, NY, USA). Statistical significance was set at  $P < 0.05$  (2-sided).

## Results

### Nestling diet and carotenoid content in livestock tissues

Cinereous vulture nestlings fed primarily on carcasses of pigs and poultry from intensive farms, wild lagomorphs, especially wild rabbits, extensive livestock, and wild ungulates. We also found scarce remains of wild birds and reptiles, as well as marine fishes obtained from rubbish dumps (Figure 2). The griffon vulture exploited livestock carcasses almost exclusively, especially from pigs and poultry; the remains of wild vertebrates were rarely found in the nests and the nestling vomits, including birds, lagomorphs, and wild ungulates. The Egyptian vulture fed on a comparatively higher variety of carcasses of medium-sized wild animals and larger carcasses of livestock. Among wild vertebrates, the proportion of

reptiles, lagomorphs (especially rabbits), other mammals, and birds was similar, while freshwater fishes were consumed in a low proportion (Figure 2). A small proportion of the remains included in the “others” category include rubbish from domestic waste dumps, as well as remains of unidentified livestock.

A literature review of carotenoid (lutein and  $\beta$ -carotene) concentrations in various tissues of livestock is shown in Table S1. These data indicate that pig, goat, sheep, and rodents generally do not incorporate carotenoids or accumulate white fat with carotenoids at very low levels, while cattle, equines, and, especially, poultry had carotenoid contents at higher concentrations depending on the tissue.

### Vegetal matter and vertebrate feces in nests, pellets, and vomits

The presence of fresh plant remains found in the nests during banding of the nestlings was anecdotal in the case of the cinereous vulture, as it was only a few remains of grasses (primarily *Stipa* spp.) in a small proportion of nests. In the

Egyptian vulture nests, no fresh plant remains were found. Bread remains were found in three nests, as well as other domestic waste items found in small numbers in a low proportion of nests of this species (Table 1). In griffon vulture nests, the presence of fresh plant remains was more frequent, especially those that usually form part of the nest structure and inner lining (genus *Cytisus*, *Quercus*, *Thymus*, *Lavandula*, *Stipa*), while fresh grasses (Poaceae), herbs of several families, and unidentified moss used to line the nests could also be potentially provisioned for consumption by the nestlings.

Plant remains consumed by nestlings were found in a proportion of the pellets and among the remains of food vomited by griffon vultures, while only rarely found in cinereous and Egyptian vulture pellets (Table 1). No remains of vertebrate excrements brought by the parents were found in the nests of cinereous and griffon vultures, and only a small proportion of canid excrements, apparently from dogs, were found in the Egyptian vulture nests (Table 1).

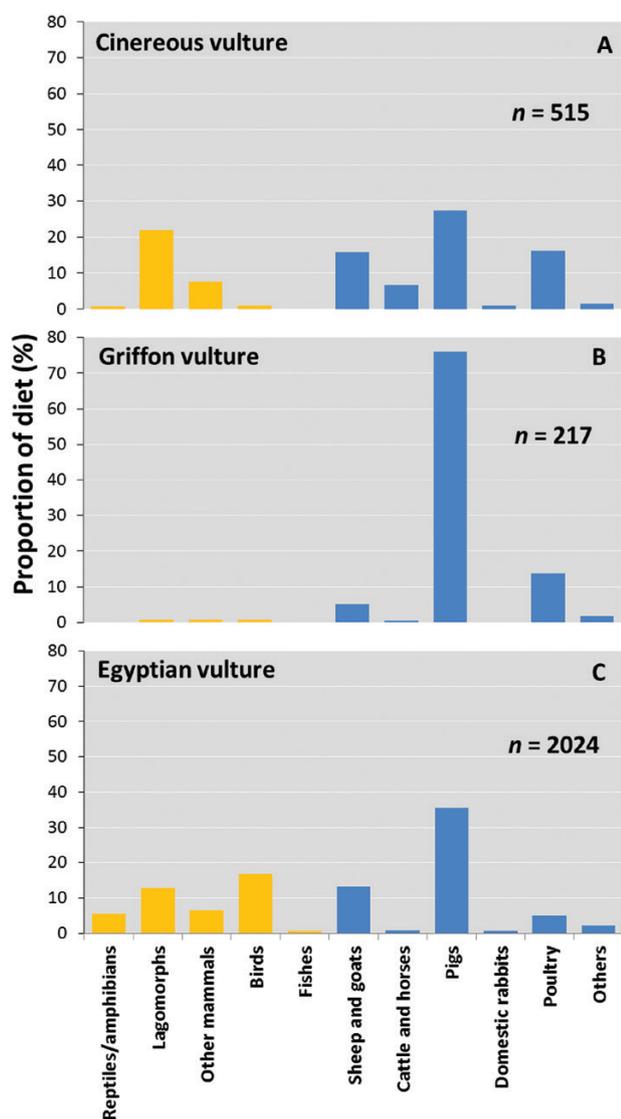
### Carotenoids in livestock feces

Chromatograms of carotenoids in cow and sheep feces had similar profiles, with three main peaks corresponding to zeaxanthin, lutein and  $\beta$ -carotene, and other minor peaks corresponding to a mixture of *cis* isomers of zeaxanthin and lutein (Figure 3). The quantification of the three main compounds present in all the samples showed much lower concentrations (mean  $\pm$  SD in  $\mu\text{g/g}$ ) with no overlapping ranges between cow and sheep feces for zeaxanthin ( $5.8 \pm 4.9$ , range = 1.8–15.6;  $50.4 \pm 7.4$ , range = 36.7–62.1), lutein ( $34.3 \pm 37.6$ , range = 8.6–107.9;  $209.4 \pm 32.9$ , range = 154.7–259.7), and  $\beta$ -carotene ( $20.9 \pm 24.2$ , range = 4.1–68.3;  $138.9 \pm 21.9$ , range = 101.2–171.8), respectively.

### Carotenoid profile and concentration in nestling blood

Plasma carotenoids identified in the three vulture species included several xanthophylls and  $\beta$ -carotene, while other minor carotenoids (canthaxanthin, lycopene, and an unidentified  $\gamma$ -carotene-like carotene) were only present in Egyptian vultures (Figure 4). Among xanthophylls, lutein, zeaxanthin, and *cis*-lutein and *cis*-zeaxanthin isomers were present in all sampled individuals of the three species (Figure 5). Plasma  $\alpha$ -cryptoxanthin and  $\beta$ -carotene were found in all sampled cinereous and Egyptian vultures, but these pigments were only found in a small proportion of griffon vultures (20% and 11.4%, respectively,  $n = 35$ ) (Figure 5). Plasma  $\beta$ -cryptoxanthin and echinenone were present in all Egyptian vultures, and in smaller proportions of cinereous and griffon vultures (Figure 5). Both lycopene and the unidentified  $\gamma$ -carotene-like carotene were only present in two siblings of Egyptian vultures.

The concentrations of each plasma carotenoid were significantly higher in the Egyptian vultures than in the other two vulture species; griffon and cinereous vultures did not differ in the concentration of any carotenoid (Table 2). The concentration of carotenoids was dominated by xanthophylls in the cinereous vulture (95%) and the griffon vulture (92%), particularly by *trans*-lutein (Table 2). In Egyptian vultures, the proportion of xanthophylls (66%) and carotenoids (34%) was more balanced. Among carotenes,  $\beta$ -carotene dominated in all species (Table 2). Overall, the total carotenoid concentration was about 8–12 times higher in Egyptian vultures than in cinereous and griffon vultures (Figure 6A). The ratio of carotenes/xanthophylls was significantly higher in Egyptian



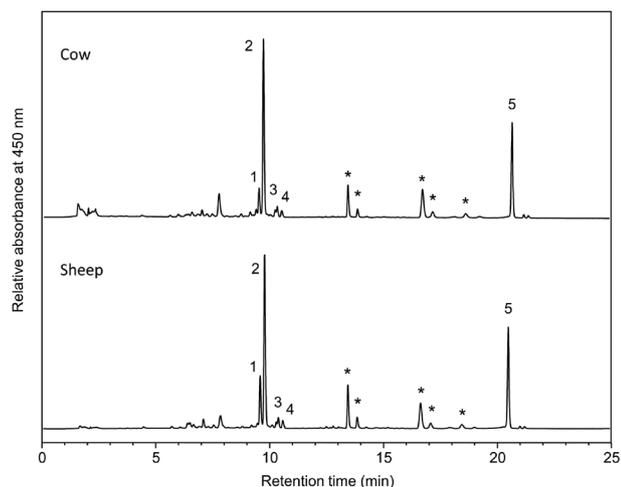
**Figure 2** Frequency (%) of remains corresponding to each category of food in the diet of nestling *Aegypius monachus*, *Gyps fulvus*, and *Neophron percnopterus* in central Spain. The bars indicate wild animal (five bars on the left) or domestic animals (five bars on the right).

**Table 1** Occurrence of vegetal matter in nest, pellets, and vomit, and presence of vertebrate feces in nests of each vulture species

Vulture species	Occurrence of vegetal matter			Occurrence of feces of food animals in nests
	% of nests ( <i>n</i> )	% of pellets ( <i>n</i> , <i>n</i> nests)	% vomit ( <i>n</i> )	% of remains ( <i>n</i> , <i>n</i> nests)
<i>Aegypius monachus</i>	9.1 (33)	1.0 (204, 39)	-	0.0 (515, 102)
<i>Gyps fulvus</i>	47.6 (63)	70.0 (60, 31)	32.7 (49)	0.0 (214 <sup>a</sup> , 311)
<i>Neophron percnopterus</i>	0.0 (40)	3.7 (21, 7)	0.0 (1)	0.2 <sup>b</sup> (2024, 138)

<sup>a</sup> Including vomit.

<sup>b</sup> Canid feces.



**Figure 3** HPLC chromatograms of carotenoids in feces from cow and sheep. Peak identities: 1, zeaxanthin; 2, lutein; 3 & 4, mixture of *cis* isomers of zeaxanthin and lutein; 5,  $\beta$ -carotene. Peaks marked with an asterisk (\*) are chlorophyll pigments (chlorophyll a and b, and derivatives). Detection wavelength was 450 nm.

vultures than in the other vulture species (ANOVA  $F_{2,49} = 48.33$ ,  $P < 0.0001$ , Figure 6B).

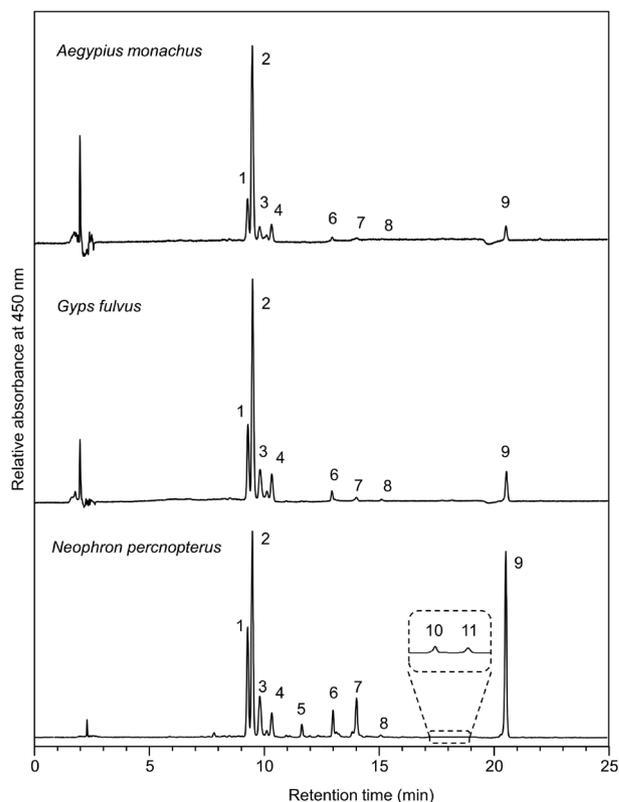
### Correlations between carotenoid concentrations and age and sex differences

The concentrations of all the plasma carotenoids found in each individual were positively correlated in the three vulture species, except for compounds found in a low proportion of individuals (e.g. echinenone in cinereous vulture, but not in griffon vultures) (Table S2). Despite being present in all individuals, echinenone was not correlated with any other compound in the Egyptian vulture.  $\beta$ -Carotene was significantly correlated with all other carotenoids in griffon and Egyptian vultures (except with canthaxanthin), but with no other carotenoid in the cinereous vulture (Table S2).

Because the concentrations of the main carotenoids were correlated (see above), we pooled xanthophylls, carotenes, and total carotenoids to analyze the variation in concentration according to nestling age (wing length) and sex. The results showed no significant differences in the concentration of xanthophylls, carotenes, and total carotenoids with age and sex in any of the three vulture species (all  $P > 0.07$ ; Table S3).

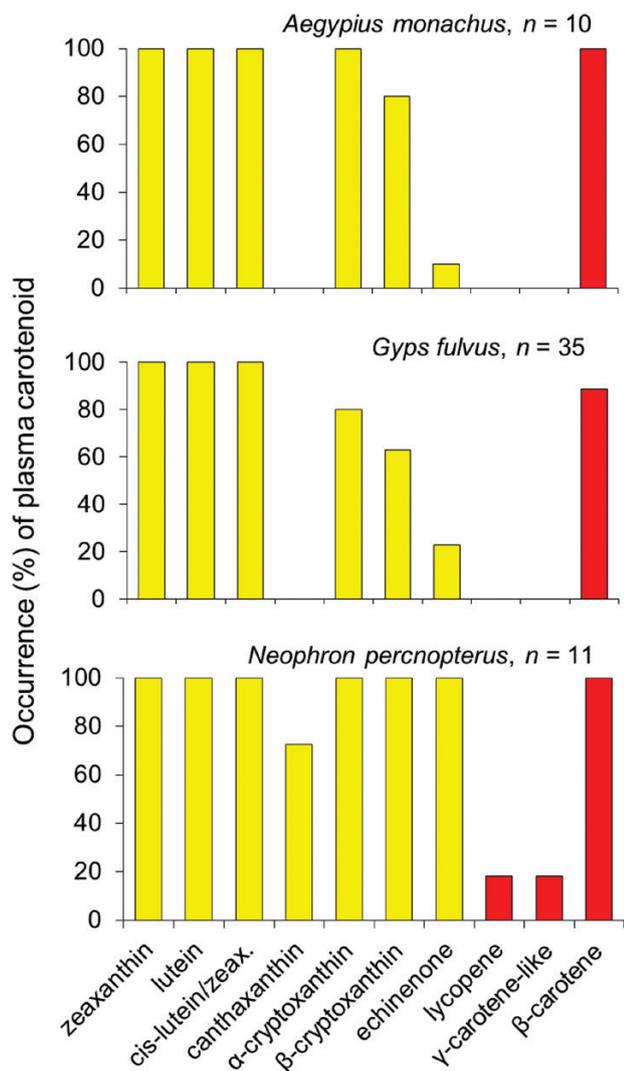
## Discussion

We found significant differences among vulture species in the relative contribution of each type of food to nestling diet. In



**Figure 4** HPLC chromatograms of plasma carotenoids from *Aegypius monachus*, *Gyps fulvus*, and *Neophron percnopterus* nestlings. Peak identities: 1, zeaxanthin; 2, lutein; 3 & 4, mixture of *cis* isomers of zeaxanthin and lutein; 5, canthaxanthin; 6,  $\alpha$ -cryptoxanthin; 7,  $\beta$ -cryptoxanthin; 8, echinenone; 9,  $\beta$ -carotene; 10, lycopene; 11, unidentified (tentatively as  $\gamma$ -carotene-like pigment). Peaks 10 and 11 were only detected in 2 Egyptian vulture individuals. Detection wavelength was 450 nm.

the study area, griffon vultures were almost entirely dependent on livestock carcasses, primarily swine and poultry. Nestling Egyptian vultures fed on a wide variety of remains from all wild vertebrate groups, especially birds, lagomorphs, and reptiles, as well as on livestock carcasses. The diet of cinereous vultures can be considered intermediate between the other species, as it includes wild mammals, especially lagomorphs, and several livestock and wild ungulates species. These results agree with general among-species differences in diet in other regions (Donázar 1993; Blanco et al. 2019), as a general consequence of the contrasting foraging ecology of the three vulture species (Donázar 1993; van Overveld et al. 2020). Diet composition can partially explain the different among-species profiles and concentrations of carotenoids in



**Figure 5** Occurrence of particular carotenoids in nestlings of each vulture species, expressed as the proportion (%) of nestlings in which each compound was found.

vulture nestling plasma (Blanco et al. 2014). In particular, the greater diversity of wild prey and their higher proportion compared to livestock could be behind the higher concentrations of carotenoids in Egyptian vultures, the species with carotenoid-dependent coloration, although only during adulthood. However, the differences in diet composition between cinereous and griffon vultures do not translate to different carotenoid profiles.

Our literature review agrees with the classification of livestock in two distinctive groups regarding the accumulation of carotenoids in adipose tissue (Schweigert 1998), although the levels of carotenoids present in the different tissues available as carrion for vultures may depend on several factors such as livestock diet, age, and on-farm carotenoid supplementation. These levels refer to fresh tissue, whereas vultures always consume this material after the death of the livestock, which means that the different states of decomposition of the tissues will determine the stability and bioavailability of the different nutrients (including carotenoids), although always in lower concentrations than in fresh tissue. Therefore, knowing the actual availability of carotenoids in vulture food is a challenging key issue that requires further research.

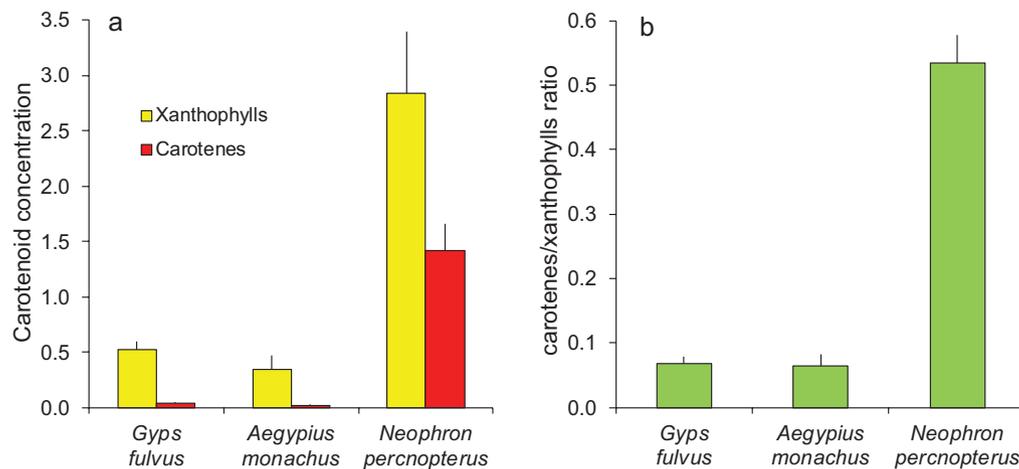
Among the circulating carotenoids found in Egyptian vultures, the occurrence of canthaxanthin, lycopene, and an unidentified  $\gamma$ -carotene-like in low concentrations is remarkable because they are not found in nestlings of the other vulture species. These pigments have not been previously found in full-grown Egyptian vultures (Negro et al. 2002), griffon vultures (Lopez-Rull et al. 2015), Andean condors, and American black vultures *Coragyps atratus* (Blanco et al. 2013). The occurrence of circulating canthaxanthin in nestling Egyptian vultures may derive from the consumption of birds, crustaceans, reptiles, and fishes with red coloration (Esatbeyoglu and Rimbach 2017). In the study area, several bird species consumed by Egyptian vultures can be dietary sources of this compound, including the red-legged partridge *Alectoris rufa*, the red-billed chough *Pyrrhocorax pyrrhocorax*, several species of finches, and probably other species with red coloration (Stradi et al. 1997, Tella et al. 2004, García-de Blas et al. 2013, authors unpubl. data). Consumption of crustaceans in the study area has only recorded shrimps obtained from a rubbish dump, although it was only detected in one year in a single nest. In addition, canthaxanthin may derive from the consumption of insects (Tyczkowski et al. 1988) and poultry, as this compound is frequently used as a feed additive in factory farms to give red color to skin and yolks (Breithaupt 2007, 2008; Esatbeyoglu and Rimbach 2017). Lycopene may derive from the consumption of wild hips and berries (e.g. several *Rosa* species in the study area) (Hornero-Mendez and Minguez-Mosquera 2000; Delgado-Pelayo and Hornero-Mendez 2012) and cultivated vegetables (e.g. tomatoes) (Dias et al. 2018) and their residues found in waste dumps, while the origin of the unidentified  $\gamma$ -carotene-like pigment remains unknown. The levels of total carotenoids found in Egyptian vulture nestlings were lower than those found in adults from the same area, but higher than in those from areas with lower primary productivity (Negro et al. 2002). Among-areas variability in circulating levels in adult Egyptian vultures has been explained by less access to ungulate feces in pastures where free-grazing livestock are less abundant, although the species being farmed is also important as there was a much higher concentration of carotenoids in sheep than cow feces, and the amount of poultry in the vultures' diet was also shown to be important. Similar among-areas differences are expected regarding circulating levels of carotenoids in nestlings depending on diet composition, which is worthy of further investigation.

The consumption and presence of fresh vegetal matter in vulture nests have been recorded previously (Kushwaha 2016), but there is no information on their frequency as a potential matter ingested by nestlings. Here, we found that fresh vegetal matter provided by parents was rare in the nests of Egyptian and cinereous vultures during the sampling snapshot period (see also Mishra et al. 2017), whereas it was comparatively frequent in griffon vulture nests. This is in agreement with the general scarcity of plant remains found in the pellets of Egyptian and cinereous vultures. In contrast, the presence of fresh remains of grasses and herbs provided by parents to the nests is apparently related to their presence in nestling vomit and pellets. The plant material vomited by nestling griffon vultures likely came from the content of the digestive tract of the carcasses of livestock which constitute their main food (Donazar 1993). In addition, some fresh plant material brought to the nests by the parents was found in a recognizable, not digested, state in some of the

**Table 2** Carotenoid concentrations (mean  $\pm$  SD  $\mu\text{g/mL}$ ) of each pigment and total carotenoid concentrations in plasma of nestling cinereous, griffon, and Egyptian vultures

Pigment	Aegyptius monachus	Gyps fulvus	Neophron percnopterus	Statistical test ( <i>P</i> )
	<i>n</i> = 10	<i>n</i> = 35	<i>n</i> = 11	
<b>Xanthophylls</b>				
Zeaxanthin	0.079 $\pm$ 0.082 <sup>a</sup>	0.131 $\pm$ 0.105 <sup>a</sup>	0.629 $\pm$ 0.427 <sup>b</sup>	20.802 (< 0.001)
Lutein	0.191 $\pm$ 0.222 <sup>a</sup>	0.256 $\pm$ 0.234 <sup>a</sup>	1.212 $\pm$ 0.777 <sup>b</sup>	17.620 (< 0.001)
<i>cis</i> -lutein/ <i>cis</i> -zeaxanthin	0.065 $\pm$ 0.062 <sup>a</sup>	0.118 $\pm$ 0.101 <sup>a</sup>	0.631 $\pm$ 0.373 <sup>b</sup>	27.223 (< 0.001)
Canthaxanthin	-	-	0.080 $\pm$ 0.114	-
$\alpha$ -Cryptoxanthin	0.010 $\pm$ 0.010 <sup>a</sup>	0.012 $\pm$ 0.015 <sup>a</sup>	0.149 $\pm$ 0.094 <sup>b</sup>	25.142 (< 0.001)
$\beta$ -Cryptoxanthin	0.004 $\pm$ 0.004 <sup>a</sup>	0.003 $\pm$ 0.004 <sup>a</sup>	0.110 $\pm$ 0.148 <sup>b</sup>	26.022 (< 0.001)
Echinenone	0.0002 $\pm$ 0.00 <sup>a</sup>	0.001 $\pm$ 0.002 <sup>a</sup>	0.029 $\pm$ 0.015 <sup>b</sup>	35.934 (< 0.001)
<b>Carotenes</b>				
Lycopene	-	-	0.045 $\pm$ 0.101	-
Unidentified ( $\gamma$ -carotene-like)	-	-	0.016 $\pm$ 0.037	-
$\beta$ -Carotene	0.019 $\pm$ 0.027 <sup>a</sup>	0.044 $\pm$ 0.057 <sup>a</sup>	1.371 $\pm$ 0.774 <sup>b</sup>	26.688 (< 0.001)
Carotenoids (total)	0.367 $\pm$ 0.401 <sup>a</sup>	0.565 $\pm$ 0.505 <sup>a</sup>	4.273 $\pm$ 2.601 <sup>b</sup>	31.191 (< 0.001)

Comparison between species was conducted with one-way ANOVAs (zeaxanthin, lutein, *cis*-lutein/*cis*-zeaxanthin, and total carotenoids) or Kruskal–Wallis test (the remainder compounds) as appropriate. Means with different letters indicate significant differences ( $P \leq 0.001$ ) between the concentration of each carotenoid according to a post hoc Tukey test for ANOVA, or Mann–Whitney test for Kruskal–Wallis test.

**Figure 6** Plasma concentrations (mean  $\pm$  SD  $\mu\text{g/mL}$ ) of (A) each carotenoid and (B) the ratio of carotenes/ xanthophylls in nestlings of each vulture species.

vomits examined. This confirms the active consumption of fresh plant material by nestling griffon vultures, provided by their parents or growing in the vicinity of the nest, especially when placed on uncovered ledges (Kushwaha 2016, authors' personal observations). The woody plants forming the nest lining may instead be contributed by the parents as part of maintaining nest hygiene, as is the case of griffon vultures (Fernández and Fernández 1974; Xirouchakis and Mylonas 2007), but is rarely provided to the nests by cinereous and Egyptian vultures, at least in the last stage of nestling rearing.

Overall, our results are in agreement with the main prediction of the “pigment availability” hypothesis, as a greater variety of compounds at higher concentrations were found in Egyptian vultures, the species with the most varied diet and the highest circulating carotenoid levels. Although full-grown individuals ingest carotenoid-rich herbivore feces (Negro et al. 2002), we found no evidence of the consumption of this

material by nestlings, and only scarce remains of uneaten canid feces were found in a low proportion of nests. Ungulate feces available in the study area are rich in carotenoids such as lutein and  $\beta$ -carotene, especially in sheep feces as compared with cow feces. We cannot rule out that the parents are providing this type of material in the crop and that the nestlings are consuming it without leaving a trace (i. Zuberogoitia, pers. com.). However, in Egyptian vultures, parents do not appear to feed their nestlings this way, but rather they carry food externally in their beaks to feed their nestlings (Elósegui 1989; Donazar 1993). These results suggest that the variety and concentration of carotenoids compounds are higher in this species due to their more varied diet, especially owing to the consumption of remains from a high variety of wild animals rather than by feeding on fecal ungulate matter rich in carotenoids. However, since all food consumed by the three species of vultures are caroteneid poor, except the vegetal

matter (Britton and Khachik 2009), the interspecific diet differences could be considered insufficient to explain the highest levels of carotenoids alone, and the exclusive presence of some compounds, in the Egyptian vulture. The infrequent occurrence of lycopene and an unidentified  $\gamma$ -carotene-like suggest the exploitation of vegetal matter that left no identifiable unconsumed remains in the nest. In the case of griffon vultures, the consumption of green plant material does not result in especially high levels of carotenoids when compared to those found in cinereous vulture that apparently does not consume this matter. Levels of circulating carotenoids in the cinereous vulture could be offset by the consumption of wild lagomorphs and other wild species, which are consumed at much lower frequency by the griffon vulture in the study area, although it consumes the vegetal content from the digestive tract of livestock carcasses. While the semi-digested vegetal content from herbivore livestock digestive tract may contribute carotenoids to the vultures diet (Blanco et al. 2014), the main consumption of pigs fed on industrial feed in the study area may limit the acquisition of these compounds compared to their potential availability in the consumption of wild and domestic herbivorous ungulates, which was the main diet of griffon vultures in the past (Donázar 1993; Blanco et al. 2019). This could contribute to the need for an extra supply of green plant material provided by the parents to the nestlings. Although this apparently does not translate into an elevated concentration of circulating carotenoids, the levels in nestling griffon vultures were clearly higher than those found in adults from the same area (López-Rull et al. 2015), but much lower than those of adult Andean condor consuming vegetal matter (Blanco et al. 2014). In the latter species, a potential relationship between circulating carotenoids and immunity was found in rehabilitated individuals suffering different pathologies (Plaza et al. 2020a), but not in apparently healthy wild individuals with higher circulating carotenoid levels due to access to wild food and vegetal matter (Blanco et al. 2013). In griffon vultures, a previous study suggested a differential carotenoid allocation to immunity in nestlings compared with full-grown individuals, as well as a higher occurrence of disease in nestlings irrespective of carotenoid levels (López-Rull et al. 2015), but this was attributed to contamination with food-borne livestock pharmaceuticals (Blanco et al. 2016, 2017). Further research is required to understand the role of the consumption of green plants linked to carotenoid acquisition and other health-related functions in griffon vultures and other avian scavengers.

Finally, our results agree with the main prediction from the “pigment assimilation-signaling” hypothesis, as we found a comparatively high variety and concentration of circulating carotenoids in nestling Egyptian vultures without carotenoid-based coloration, even when they are largely dependent on livestock carcasses with presumably low levels of these compounds (Álvarez et al. 2015; Green and Fascetti 2016). Despite greater dietary diversity, most of the wild food consumed by Egyptian vultures is derived from animals that have been dead for a variable period of time, which may mean that much of the carotenoids originally present are less bioavailable due to degradation as a result of putrefaction of the tissues containing them. This suggests that, compared with cinereous and griffon vultures, this species is especially efficient in uptaking the different carotenoids during digestion, even when they were present in low concentrations in their food. The fact that these carotenoids were only found in Egyptian

vultures suggests that the other study species have a less-efficient metabolic machinery to process them, rather than due to the absence of these pigments in their diet. In particular, the occurrence of circulating canthaxanthin would be expected in cinereous and griffon vultures due to the consumption of poultry often supplemented with this and other carotenoids, as both species feed on their carcasses at more than twice the proportion found for the Egyptian vulture. However, this needs to be confirmed in the study area, as supplementation with canthaxanthin likely does not occur in all farms. An experimental supplementation approach could elucidate whether vulture species other than Egyptian vulture have the capacity to assimilate this and other carotenoids. The apparent lack of parental provisioning to nestling Egyptian vultures with unusual sources of carotenoids, such as ungulate feces, agrees with their lower levels of total carotenoids compared with adults from the study area (Negro et al. 2002), which is consistent with a higher demand of these compounds for signaling coloration in adults (van Overveld et al. 2020). Instead, feeding with low nutritive feces could be costly for nestlings with an immature immune system by exposing them to gastrointestinal pathogens (Negro et al. 2002; Plaza et al. 2020b), and by the potential dysbiosis of their protective gut microbiota. Because carotenoids are apparently not deposited in the integument of nestlings, they could be destined to other functions related with health and development, which is worthy of further research.

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## Author Contributions

G.B and D.H-M. conceived the study; G.B. undertook the fieldwork; D.H-M. conducted the laboratory analyses; G.B and D.H-M. wrote the manuscript.

## Ethics Statement

Our study followed the ethical guidelines proposed by the Spanish Royal Decree 1205/2005 on the protection of animals used in experiments and scientific research. This was carried out in accordance with permits from the Spanish Bird Ringing Centre (permit number: 530115 to GB) and the regional government of Castilla y León (expedient, EP/CyL/282/2013), Dirección General del Medio Natural, Servicio de Espacios Naturales.

## Conflict of Interest statement

The authors declare no conflict of interests.

## Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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